# Genetic elimination of CCR2 provides minimal protection of bone functional capacity

A THESIS
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF ARTS

Moira Petit

August 2011

## Acknowledgements

The author would like to thank Dawn Lowe and Gordon Warren for their assistance with writing this thesis; Angela Lin, Robert Guldberg, Susan Novotny, Sarah Greising, and Kristen Baltgalvis for their assistance with interventions and testing.

The author would like to acknowledge NIH grant R01 AG031743 for support of this work.

**Abstract**

Chemokines are proposed to be involved with bone loss. The purpose of this study was to determine whether genetic elimination of C-C chemokine receptor 2 (CCR2) was protective of bone geometry and function. Ovariectomy and denervation were used as interventions to induce bone loss in mice with and without CCR2 (C57BL/6 wild type and CCR2-knockout ($CCR2^{-/-}$) mice, respectively). Cortical geometry and trabecular morphology of tibial bones were determined using μCT analyses. Bone mechanical properties were calculated from load displacement curves using 3-point bending testing. Overall, the elimination of CCR2 had a minor protective effect on bone mechanical properties. $CCR2^{-/-}$ mice had bones slightly larger and stronger than C57BL/6 mice. In ovariectomized and denervated mice, $CCR2^{-/-}$ tibiae exhibited 5-18% greater cross sectional area, cross sectional moment of inertia, ultimate load, and stiffness than C57BL/6 tibiae indicating a resistance to intervention-induced bone loss in the $CCR2^{-/-}$ mice. Trabecular bone volume fraction and bone mineral density was 2-22% greater in CCR2-/- mice than C57BL/6 mice at both the epiphysis and the metaphysis. Additionally, periosteal diameter was protected in $CCR2^{-/-}$ mice but not in C57BL/6 mice with interventions. It is concluded that genetic elimination of CCR2 results in larger bones that are minimally protected under conditions of induced bone loss.

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## Introduction

Bone loss is a common problem affecting elderly and injured persons. This study investigated the role of C-C chemokine receptor 2 (CCR2) as a possible mediator of bone loss. CCR2 is one in a family of 11 chemokine receptors. In bone, it is primarily expressed on preosteoclasts and osteoclasts, cells that are responsible for bone resorption [1, 2]. Theoretically, if the action of CCR2 is mitigated, bone loss would be blunted. In support of this, a previous study by Binder et al. reported that CCR2 knock-out ($CCR2^{-/-}$) mice have impaired osteoclast development and decreased bone resorption [3]. The study directly compared wild type mice to $CCR2^{-/-}$ mice under control conditions and a model of ovariectomy-induced bone loss. Binder and colleagues reported that the serum concentration of deoxypyridinoline, a marker of bone resorption, was low in $CCR2^{-/-}$ mice, relative to wild type mice under control conditions, while there was minimal effect on the serum concentration of osteocalcin, a marker of bone growth. Because bone was resorbed at a decreased rate in $CCR2^{-/-}$ mice, and the rate of growth remained constant, Binder and colleagues asserted that $CCR2^{-/-}$ mice appeared to be protected from the bone loss that occurs during bone remodeling.. Additionally, the study found that $CCR2^{-/-}$ mice were protected from systemic bone loss induced by ovariectomy. Binder et al considered only several properties of bone and focused on vertebra and not long bones. Additionally, the study used only the ovariectomy model of bone loss.

The current study looks to expound on the findings of Binder and colleagues by using tibial bones to consider structural properties of cortical and trabecular bone as well as functional properties under ovariectomy and denervation models of bone loss. Estrogen deficiency is the main cause of osteopenia in post-menopausal women, and estrogen has been shown to play an important role in the regulation of bone homeostasis, in part through the immune system by way of cytokines, chemokines, and their receptors such as CCR2 [4, 5]. Denervation involves disruptions in bone homeostasis patterns similar to those seen with disuse or when normal loading is removed from the bone. We hypothesized that under the two different means of inducing bone-loss, structural and functional properties of long bones from $CCR2^{-/-}$ mice would be less detrimentally affected than wild type mice.

## Methods

*Animals-*

CCR2 $^{-/-}$ mice on a mixed C57BL/6 x strain 129 genetic background were generated and then backcrossed to C57BL/6 mice for 10 generations as previously described. C57BL/6 mice were obtained from Jackson Laboratory. All mice were female and 20 weeks old at the start of the study. Mice were provided phytoestrogen-free rodent chow (2019 Teklad Global 19 Protein Rodent Diet, Harland 99 Teklad, Madison, WI) and water ad libitum. Animals were housed 4-5 per cage and maintained on a 12-12 hour light-dark cycle.

For surgical interventions, mice were anesthetized using an induction chamber with isoflurane and then maintained using inhalation of 1.75% isoflurane mixed with O2 at a flow rate of 200 ml/min. A sterile environment was maintained during all surgeries. Immediately following surgery, mice were observed for return to normal ambulation and given an subcutaneous injection of 0.15 μg of buprenorphine as an analgesic. Mice were assessed daily for seven days following procedures. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

*Study Design:*

Mice were randomly assigned to one of three intervention groups: control, ovariectomy, or denervation.. Eight weeks post intervention, mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (100mg/kg). Tibial bones were removed

with precautions taken to keep the growth plate intact, and stored in phosphate-buffered saline at -20°C until time of μCT analyses. Uteri of control and ovariectomized mice were removed and weighed to confirm effectiveness of ovariectomy intervention. Mice were then euthanized via exsanguination.

*Surgical Interventions:*

For the bilateral ovariectomy intervention, A dorsal skin incision was made between the iliac crest and lower ribs. The ovaries were accessed through two abdominal muscle wall incisions. The ovarian duct and artery were cauterized prior to removal of the ovaries. The abdominal wall incisions were sutured with 6-0 silk suture and 7-mm wound clips were applied to the dorsal skin incision. The procedure has been further described in previous studies [6, 7].

For the unilateral denervation intervention, the left tibial nerve was transected. The procedure has been described previously [7]. In brief, the mouse was positioned in right lateral recumbency, and the left foot was stabilized for the duration of the surgery. A 15mm skin incision was made parallel and slightly posterior to the femur. The tibial nerve was accessed through an incision in the biceps femoris muscle. A 7-0 sterile silk suture was used to make a knot on the most distal portion of the tibial nerve. The nerve was cut distal to the knot , retracted, cut as proximal as possible, and removed. The muscle and skin were closed using 6-0 silk suture.

*Bone Analysis:*

For the μCT analysis, volumes of interest (VOIs) in the mid-diaphysis and proximal tibiae were scanned with a μCT 40 (Scanco Medical, Brüttisellen, Switzerland) at a voltage of 55kVp, current of 145μA, integration time of 200ms, and voxel size of 12μm. Global constant thresholding values to segment bone from background were chosen based on histogram analysis and visual inspection. These segmentation values were applied to generate 3D binarized images which were used to quantify bone morphometry and density. Analyses were performed to determine cortical parameters for the mid-diaphysis (mineral density, cortical thickness, minimum cross sectional moment of inertia, and cross sectional area). In the proximal tibia, trabecular bone at the epiphysis and the metaphysis was assessed at 0.24mm and 0.60mm from the growth plate, respectively, for mineral density, bone volume fraction, trabecular number and trabecular thickness. Morphometric parameters were computed using direct distance transformation methods [8].

Three-point bending at mid-diaphysis on a Mecmesin MultiTest 1-D test machine using a Mecmesin AFG-25 load cell (Mecmesin, West Sussex, UK) was used to determine tibial bone functional properties. Tibial length was measured with digital calipers and the mid-diaphysis of the tibia was identified. The tibia was placed onto a set of supports separated by 1 cm such that the lateral side of the bone was placed downward. Bones were placed in this position to provide the most stable loading position possible during the testing procedure. Quasi-static loading was applied to the central aspect of the bone on the

medial surface using a displacement rate of 2mm/min. The load applied to the bone measured by a load cell with 5mN resolution. The load and displacement outputs were sampled at 14 Hz by a computer and software (TestPoint version 7; Measurement Computing Corp., Morton, MA). The procedures used to assess the functional capacities of the mouse tibia have been described in detail previously [8, 9].

The load-displacement curve for each bone was analyzed using a custom-written TestPoint program. The functional measure of tibial bone were quantified by ultimate load, stiffness, and deflection and energy absorbed to ultimate load. Ultimate load was determined by the program as the highest load obtained prior to failure. Once the point corresponding to ultimate load was determined on the load-displacement curve, deflection and energy to ultimate load were determined. Ultimate stress and modulus of elasticity were calculated using classical beam theory. The equations are as follows: Ultimate Stress=$(UL\times d\times L)/(8\times CSMImin)$ and Modulus of Elasticity=$(k\times L^3)/(48\times CSMImin)$ where UL is ultimate load, d is periosteal diameter, L is bottom support span length, and k is stiffness [8, 9].

*Statistical Analyses:*

2-way ANOVAs were performed (genotype x intervention) to analyze the effects of genotype and intervention on bone parameters. All statistical analysis were done using SigmaStat version 3.5 (Systat Software). Significance was accepted with an $\alpha$ level of 0.05. Multiple regressions (genotype x body mass) were run on parameters with

significant genotype main effects to determine if the main effect was due to genotype or was a result of a difference in body mass between the genotypes.

## Results

*Body mass*

To determine if mouse size differed between C57BL/6 and CCR2 $^{-/-}$ mice, or if interventions affected these mice differently, body masses pre and post interventions were analyzed. At the start of the study, CCR2 $^{-/-}$ mice weighed more than C57BL/6 mice (22.9± 0.5 vs. 21.0± 0.5g, respectively, $p<0.001$). CCR2$^{-/-}$ mice had 17% greater body mass at the end of the study compared to C57BL/6 mice (28.9±0.5 vs. 24.6±0.5g, respectively, $p<0.001$). At the end of the study, ovariectomized mice weighed more than control and denervated mice (30.1±0.7, 25.3±0.6, and 24.8±0.6 g, respectively, $p<0.001$), regardless of genotype (interaction, $p= 0.748$).

*Genotype and intervention affect bone mechanical properties*

Tibial ultimate load was affected by the type of intervention, for both C57BL/6 and CCR2 $^{-/-}$ mice ($p<0.001$). Tibia from denervated mice had ultimate loads 7-15% lower than those from control mice (Figure 1, left). Tibial ultimate load was also affected by genotype ($p=0.002$); however, results of a multiple regression determined the genotype effect was due to the difference in body masses between genotypes and not due to a protective effect of bone offered by either genotype. There was an effect of genotype and intervention on tibial stiffness ($p=0.022$ and 0.031, respectively) trending towards a significant interaction. Once again, a multiple regression determined that the genotype effect was due to a difference in body masses. For C57BL/6 mice, interventions resulted

in 9-14% lower tibial stiffness. Among the CCR2$^{-/-}$ mice, ovariectomy intervention resulted in 5% greater tibial stiffness and denervation resulted in 2% lower tibial stiffness than control(Figure 1, right).

*Geometric determinants of cortical bone mechanical properties*

Due to the way mechanical testing was performed, both cross sectional area (CSA) and minimum cross sectional moment of inertia(CSMI) are geometric determinants of bone mechanical properties. Independent main effects of both genotype and intervention were found for both measures for C57BL/6 and CCR2 $^{-/-}$ mice (CSA genotype $p<0.001$, intervention $p<0.001$; CSMI genotyope $p<0.001$, intervention $p=0.002$) (Figure 2). Results of a multiple regression determined the genotype effect was independent of the difference in body masses between genotypes. CSA was 4-6% greater in CCR2$^{-/-}$ mice than C57BL/6 mice across all interventions. CSMI was 6-18% greater in CCR2$^{-/-}$ mice than C57BL/6 mice across all interventions. As such, a protective effect on bone exists for CCR2$^{-/-}$ mice.

*Cortical bone quality in response to intervention varies with genotype*

To determine if cortical bone intrinsic mechanical properties were affected by genotype and/or intervention type, ultimate stress and modulus of elasticity were calculated. An interaction between genotype and intervention type for ultimate stress and a genotype main effect for modulus of elasticity were detected (Table 1). Ultimate stress for C57BL/6 mice was 6% higher in ovariectomized than in control mice. For CCR2$^{-/-}$ mice,

ultimate stress did not differ between interventions. Modulus of elasticity was lower across all interventions for CCR2$^{-/-}$ compared to C57BL/6 mice, this was shown by a multiple regression analysis to be the result of genotype differences and not body mass differences. Cortical bone also showed a main effect of genotype on bone mineral density and cortical thickness(Table 1). Bone mineral density was 2% greater for CCR2-/- than C57BL/6 mice. The results of a multiple regression indicate this is due to a genotype difference and not associated with body mass. Cortical thickness was 3% greater for CCR2$^{-/-}$ than C57BL/6 mice. A significant interaction for periosteal diameter was detected. Tibial bones from denervated and ovariectomized C57BL/6 mice were 3% smaller than C57BL/6 control mice. Periosteal diameter did not differ across intervention groups for CCR2 $^{-/-}$ mice, indicating a protective effect when CCR2 is absent.

*Trabecular bone geometric measures where effect of intervention was dependent on genotype*

Significant genotype-intervention interactions were detected for several parameters of trabecular bone geometry (Table 1). A significant interaction was found for bone mineral density at the metaphysis. The ovariectomy intervention group of C57BL/6 mice had lower bone mineral density than both the denervation and control groups. For CCR2$^{-/-}$ mice, bone mineral density at the metaphysis was not different among interventions. A significant interaction existed for both the bone volume fraction and trabecular thickness at the epiphysis. Bone volume fraction was less for denervation than control in C57BL/6 mice. In CCR2$^{-/-}$ mice, there was no difference in bone volume fraction at the epiphysis

among intervention groups,. Trabecular thickness at the epiphysis was low in tibia ofC57BL/6 mice that were ovariectomized and denervated, but for $CCR2^{-/-}$ mice, only ovariectomy adversely affected trabecular thickness, showing a discrepancy in how the intervention affects trabecular thickness depending on the genotype of the mouse..

*Trabecular bone geometric parameters that were affected by genotype*

Results of μCT were compared between C57BL/6 and $CCR2^{-/-}$ mice and parameters with an effect of genotype were identified (Table 1). At the epiphysis, only bone mineral density showed a main effect of genotype with $CCR2^{-/-}$ bones being 2% denser than C57BL/6 bones. A multiple regression determined this is due to genotype differences and not body mass. At the metaphysis, there were genotype differences in both bone volume fraction and trabecular thickness. Bone volume fraction was 22% higher for $CCR2^{-/-}$ than C57BL/6 mice when combined across all intervention groups. Trabecular thickness was 8% greater in $CCR2^{-/-}$ mice than C57BL/6 mice. The results of multiple regressions found both the above to be a result of genotype differences not associated with body mass.

*Bone geometric parameters that were only affected by interventions*

In trabecular bone, there were intervention affects at both the metaphysis and the epiphysis. At the metaphysis, bone volume fraction and trabecular number were lower than control in ovariectomized and denervated $CCR2^{-/-}$ and C57BL/6 mice. Trabecular thickness was lower than control in denervated and ovariectomized C57BL/6 mice, and

in ovariectomized but not denervated CCR2$^{-/-}$ mice. At the epiphysis, trabecular number was lower than control in denervated CCR2$^{-/-}$ and C57BL/6 mice, but not in ovariectomized mice. In cortical bone, denervated and ovariectomized CCR2$^{-/-}$ and C57BL/6 mice had lower cortical thickness than control mice.

Table 1: Tibial bone cortical geometry and trabecular bone morphology from mice with and without C-C chemokine receptor 2 and in response to ovariectomy and denervation interventions

| | C57BL/6 | | | $CCR2^{-/-}$ | | | P-Values | | |
|---|---|---|---|---|---|---|---|---|---|
| | Control n=10 | OVX n=10 | DEN n=10 | Control n=9 | OVX n=8 | DEN n=10 | Intervention | Genotype | Interaction |
| *Cortical geometry at mid-shaft:* | | | | | | | | | |
| Mineral Density ($mg/cm^3$) | 1443±7 | 1449±7 | 1440±7 | 1463±7 | 1472±7 | 1460±7 | 0.268 | <0.001 | 0.966 |
| Cortical thickness (mm) | 0.184±0.002 | 0.176±0.002 | 0.165±0.002 | 0.191±0.003 | 0.177±0.003 | 0.171±0.002 | <0.001 | 0.033 | 0.414 |
| Periosteal Diameter (mm) | 1.19±0.01 | 1.15±0.01$^{+}$ | 1.15±0.01$^{+}$ | 1.20±0.01 | 1.23±0.01* | 1.20±0.01* | - | - | 0.024 |
| *Cortical intrinsic mechanical properties:* | | | | | | | | | |
| Ultimate Stress (MPa) | 273.0±5.8 | 289.8±5.8 | 266.6±5.8 | 276.1±6.1 | 266.5±6.5* | 272.2±5.8 | - | - | 0.037 |
| Modulus of Elasticity (GPa) | 13.1±0.3 | 12.8±0.3 | 13.3±0.3 | 12.0±0.4 | 12.3±0.4 | 12.4±0.3 | 0.653 | 0.005 | 0.7054 |
| *Trabecular morphology at metaphysis:* | | | | | | | | | |
| Mineral Density ($mg/cm^3$) | 1222±5 | 1197±5$^{+}$ | 1226±5 | 1224±5 | 1233±6* | 1240±5 | - | - | 0.009 |
| Bone Volume/ Total Volume | 0.040±0.002 | 0.024±0.002 | 0.020±0.002 | 0.048±0.002 | 0.032±0.002 | 0.032±0.002 | <0.001 | <0.001 | 0.523 |
| Trabecular Number ($mm^{-1}$) | 3.13±0.07 | 3.06±0.07 | 2.88±0.07 | 3.10±0.07 | 2.98±0.07 | 2.99±0.07 | 0.041 | 0.993 | 0.317 |
| Trabecular Thickness (mm) | 0.050±0.001 | 0.045±0.001 | 0.047±0.001 | 0.052±0.001 | 0.050±0.001 | 0.053±0.001 | 0.002 | <0.001 | 0.210 |
| *Trabecular morphology at epiphysis:* | | | | | | | | | |
| Mineral Density ($mg/cm^3$) | 1272±4 | 1258±4 | 1265±4 | 1287±5 | 1283±5 | 1284±4 | 0.153 | <0.001 | 0.543 |
| Bone Volume/ Total Volume | 0.175±0.009 | 0.168±0.009 | 0.140±0.009$^{+}$ | 0.202±0.009* | 0.170±0.010$^{+}$ | 0.191±0.009* | - | - | 0.027 |
| Trabecular Number ($mm^{-1}$) | 76.5±6.1 | 87.4±6.1 | 63.3±6.1 | 81.5±6.4 | 78.6±6.8 | 68.4±6.1 | 0.020 | 0.933 | 0.454 |
| Trabecular Thickness (mm) | 0.057±0.001 | 0.053±0.001$^{+}$ | 0.054±0.001 | 0.059±0.001 | 0.053±0.002$^{+}$ | 0.061±0.001* | - | - | 0.014 |

* indicates different from C57BL/6 for same intervention
+ indicates different from control of same genotype

Values are normalized means ± SE. BV/TV is the proportion of the bone volume that is bone tissue. C57BL/6 are wild type mice and $CCR2^{-/-}$ are mice without c-chemokine receptor 2. OVX represents ovariectomy and DEN represents the denervation intervention.

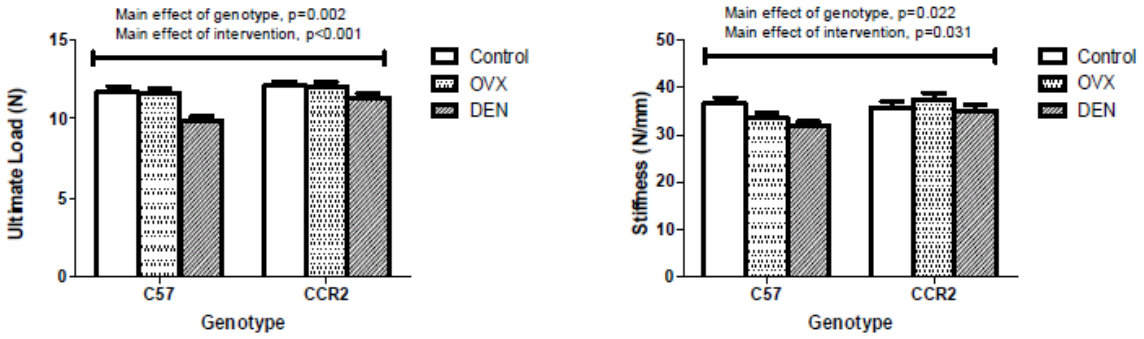


Figure 1: CCR2$^{-/-}$ mice exhibited greater resistance to induced loss of tibial mechanical properties. Left: ultimate load from mice with and without C-C chemokine receptor 2 and in response to ovariectomy and denervation interventions. Right: stiffness from mice with and without C-C chemokine receptor 2 and in response to ovariectomy and denervation interventions.

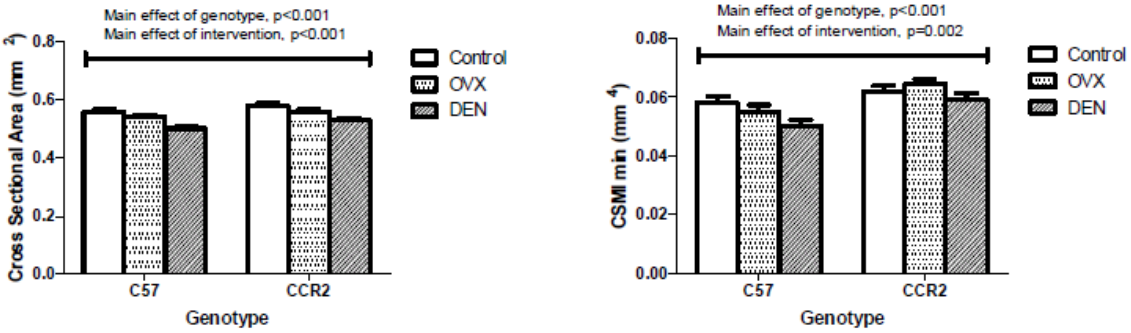


Figure 2: Denervation intervention was detrimental to geometric predictors of bone function. Left: Cross sectional area of tibiae from mice with and without C-C chemokine receptor 2 and in response to ovariectomy and denervation interventions. Minimum cross sectional moment of inertia (CSMImin) from mice with and without C-C chemokine receptor 2 and in response to ovariectomy and denervation interventions.

## Discussion

Bone loss occurs with increased age and injury. Previous studies have shown mitigation of CCR2 to play a role in diminishing bone loss under estrogen depleted conditions. The protection of bone geometry and function was attributed to decreasing the rate of bone resorption in $CCR2^{-/-}$ mice[2, 3]. These studies proposed that CCR2 could be a potential therapeutic target for bone loss due to the role it plays in regulating osteoclast function.

We hypothesized that when subjected to bone loss conditions, $CCR2^{-/-}$ mice would be less detrimentally affected and have bones that are larger and stronger than C57BL/6 mice. The results of the study are in agreement with Binder et al that $CCR2^{-/-}$ mice exhibit larger body masses and bones than C57BL/6 mice. Additionally, this study found that bones from $CCR2^{-/-}$ mice are stiffer and able to withstand a higher ultimate load than C57BL/6 mice. Thus we postulate that, since the above are indicators of the ability of a bone to withstand fracture, $CCR2^{-/-}$ mice have stronger bones than C57BL/6 mice due to a larger body mass and a protective effect of $CCR2^{-/-}$ on bone strength.

Our study found that the effects of CCR2 on bone geometry are small and do not appear to vary much from the C57BL/6 mice when subjected to bone loss interventions. Significant genotype main effects were found for many parameters. Multiple regression was used to determine if the genotype effect was a result of a body mass difference between the genotypes or if bones from $CCR2^{-/-}$ mice are more resilient when subjected

to bone loss conditions. For all the genotype main effects found for bone geometry, the results of the multiple regression indicated that the $CCR2^{-/-}$ bones were less detrimentally affected than the C57/BL6 bones.

To our knowledge, the only previous study to look at the effect of CCR2 on bone geometry and function was done by Binder and colleagues. Based on the findings of this study, the larger bones of the $CCR2^{-/-}$ mice found in the previous study are characteristic of the phenotype, as further characterized in the present study, and not in itself an indicator of a protective effect of $CCR2^{-/-}$ on bone. Because $CCR2^{-/-}$ mice are larger than C57BL/6 mice, the protective effect of CCR2 is not as great as would be suggested by what was found in the previous study. Due to the small protective effect of $CCR2^{-/-}$ on bone geometry and function, it is not possible to make any speculations about CCR2 playing a substantial role in mitigating bone loss.

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